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Curcumin – A Potent Inhibitor of Galectin-3 Expression

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Summary

The expression of galectin-3, a β-galactoside binding lectin, was found to be affected by different kinds of stressors, and is strongly modified in numerous physiological and pathophysiological conditions. Although no precise regulatory mechanisms of galectin-3 expression are unraveled, transcription factors AP-1 (activator protein 1) and NF-κB (nuclear factor kappa B) play an important role in these processes. Activities of both transcription factors are affected by curcumin, a biologically active compound extracted from rhizomes of *Curcuma* species. We have analyzed the impact of curcumin on the expression of galectin-3 in glioblastoma cells under basal conditions and under stress invoked by the cell exposure to alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and ultraviolet C (UV-C) light. Galectin-3 level was measured by western-blot technique using M3/38 monoclonal antibody. Curcumin has decreased the basal level of galectin-3, while the pretreatment of cells with curcumin has considerably reduced the inducible effect of UV-C radiation and abolished the inducible effect of alkylating agent. Thus, curcumin has been identified as a potent inhibitor of galectin-3 expression.

Key words: galectin-3, curcumin, UV light, alkylating agent MNNG

Introduction

Galectin-3, a 32 kD lectin that specifically recognizes β-galactoside structures of glycans, plays a pivotal role in several biological processes *(1)* and its ubiquitous localization (in the nucleus and cytoplasm, on the cell membrane and in the extracellular matrix) only confirms its diverse functions, including modulation of cell adhesive properties *(2–4)* and regulation of cell motility *(5)*. Galectin-3 shows anti-apoptotic and growth-enhancing properties *(6)* and can promote cell invasiveness *(7)*. These abilities of galectin-3 are closely associated with assets of tumorigenesis and metastasis (*8–10*) (*e.g.* ectopic expression or inhibition of galectin-3 has been shown either to increase or decrease tumorigenicity and metastatic proclivity of tumor cells, respectively *(11–13)*). Recently, elevation of galectin-3 level has become a useful diagnostic marker for breast carcinoma *(14)*, chordoma *(15)*, thyroid carcinoma *(16)* and some other tumors.

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadien-3,5-di-one] is a natural, biologically active compound extracted from rhizomes of *Curcuma* species that gives the golden-yellow color and unique flavor to curry. In Indian medicine it has been known for centuries, while Western medicine discovered only lately its anti-inflammatory, anti-oxidative, and cytostatic properties, as well as a potential to be used as a chemopreventive agent in humans *(17,18)*. It seems that mechanisms underlying these effects involve suppression of regulator(s) involved in activation of transcription factors c-Jun and NF-κB (nuclear factor kappa B) *(19,20)*. According to the results of our previous studies both of these factors are involved in the regulation of the basal expression of galectin-3 as well as of induced expression of galectin-3 caused by alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and ultraviolet C (UV-C)

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light (*21*). This is why both MNNG and UV-C light were used as model stressors in this study of the ability of curcumin to inhibit the expression of galectin-3.

Materials and Methods

Materials

All chemicals were of analytical grade and purchased from Sigma (St. Louis, MO, USA), if not stated otherwise. Nitrocellulose membranes were purchased from Millipore Corp. (Bedford, MA, USA), and heat-inactivated fetal calf serum (FCS) from Life Technologies (Rockville, MD, USA). Rat monoclonal antibody M3/38 (anti-galectin-3) was kindly provided by Dr. I. Rosenberg (Harvard Medical School, Boston, MA). MNNG, anti-mouse immunoglobulin G (IgG) labeled with alkaline phosphatase, and anti-rat IgG labeled with alkaline phosphatase were from Sigma (St Lewis, MO). Antibodies against c-Jun were from Oncogene Research Products (Cambridge, MA).

Cell lines, cell culture, and stress procedures

We have recently shown that human glioblastoma A1235 cells undergo prompt glycosylation changes after exposure to different stressors *(22,21)* and all experiments described here were performed on this cell-line. Cells were asynchronously grown (37 °C, 5 % CO_2, relative humidity 95 %) in the Dulbecco's modified Eagle's medium (Sigma, Cat. No. D5648) supplemented with 10 % heat-inactivated FCS, 100 μg/mL streptomycin and 100 U/mL penicillin. Cells were subcultured twice a week to maintain cultures in exponential growth.

In all experiments cells were plated in tissue culture dishes (60 × 15 mm) 24 h before treatment.

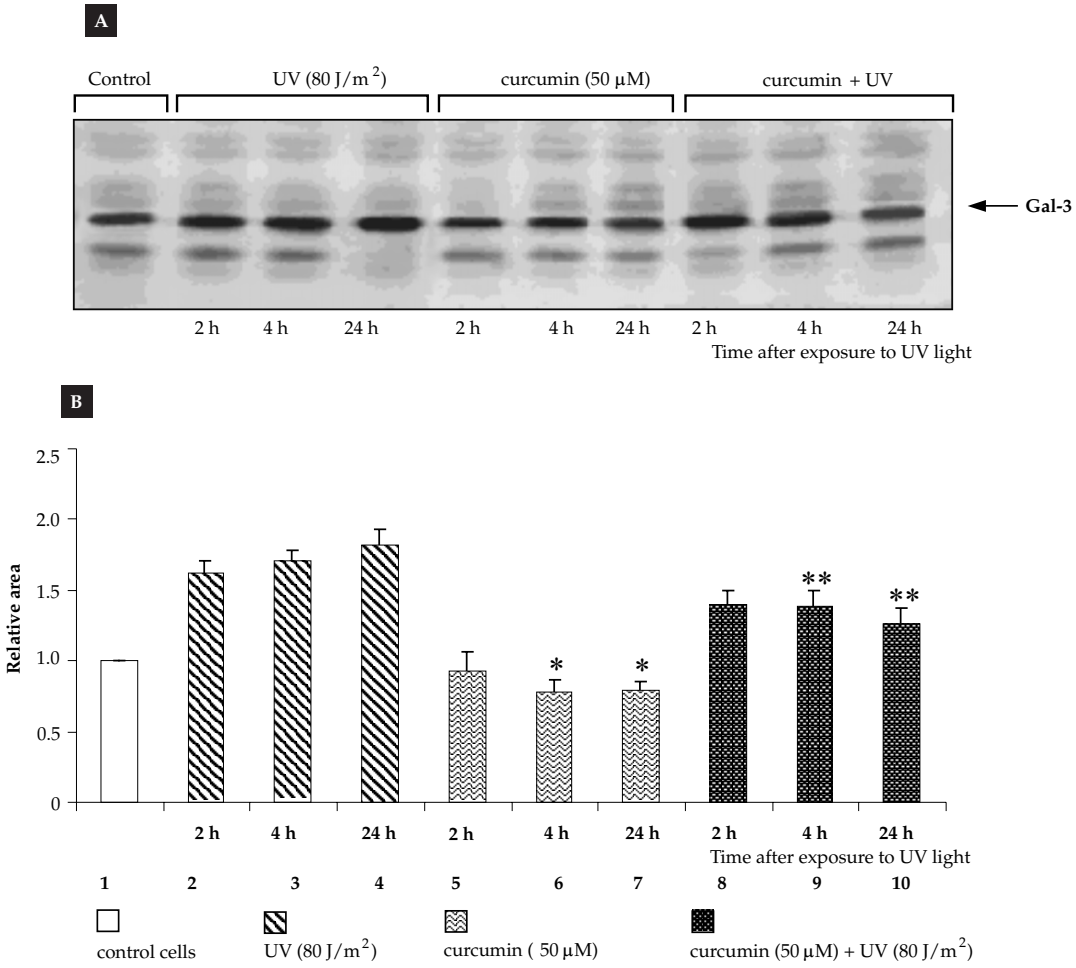

Fig. 1. Effects of curcumin on basal and UV-C-induced expression of galectin-3.
A1235 glioblastoma cells were pre-incubated for 1 h in medium with or without curcumin (50 μM), exposed or not to UV-C radiation (80 J/m^2) and analyzed for galectin-3 (Gal-3) after 2, 4 and 24 h of further cultivation. Control cells were exposed neither to curcumin nor to UV-C radiation.
A. Western blot analysis of galectin-3. Cellular proteins (10 μg per lane) were separated by 12 % SDS-PAGE, transferred to Immobilon-P membranes and analyzed with antibodies against galectin-3.
B. The amount of galectin-3 was estimated by densitometric analysis and expressed as normalized values relative to the level of galectin-3 in the control cells. Average values (± standard deviation (SD)) from two independent series of experiments (analyzed in duplicates) are shown.
* - $p < 0.05$ comparing to the controls, ** - $p < 0.05$ compared to the corresponding curcumin-untreated cells

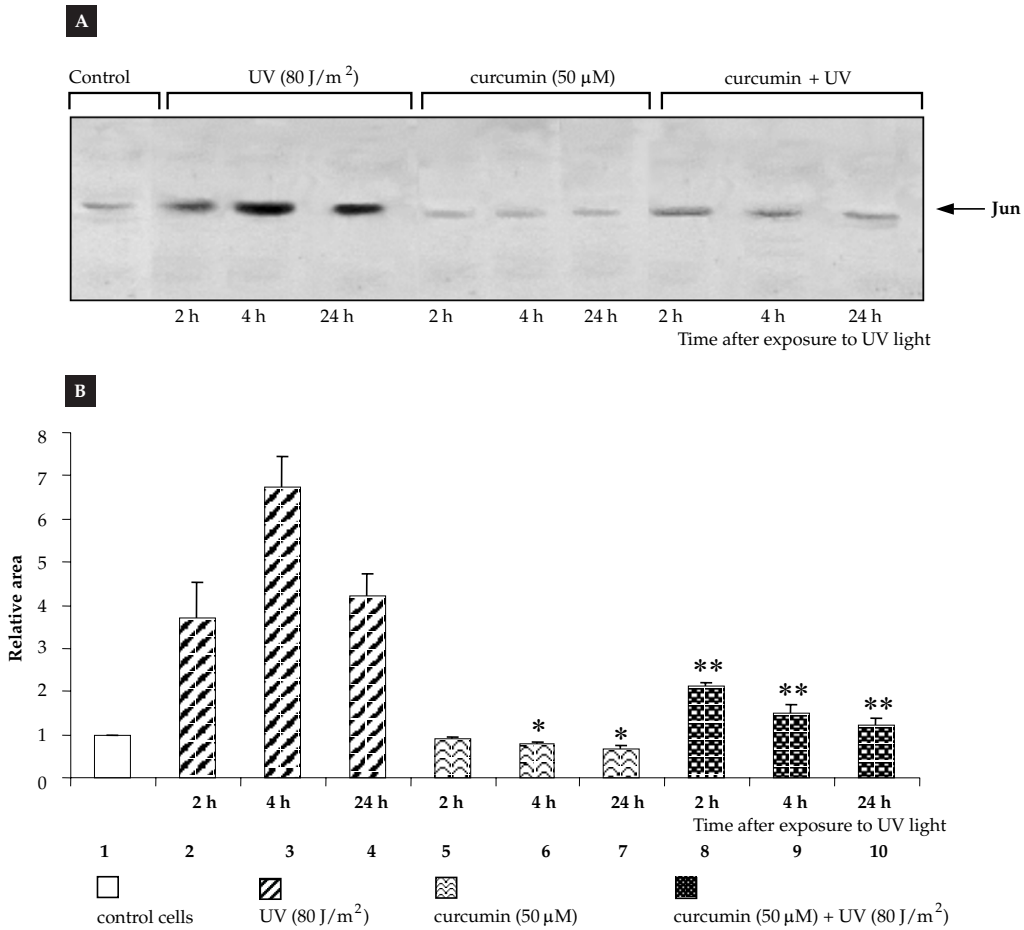

Fig. 2. Effects of curcumin on basal and UV-C-induced expression of c-Jun.
A1235 glioblastoma cells were pre-incubated for 1 h in medium with or without curcumin (50 μM), exposed or not to UV-C radiation (80 J/m^2) and analyzed for c-Jun after 2, 4 and 24 h of further cultivation. Control cells were exposed neither to curcumin nor to UV-C radiation.
A. Western blot analysis of c-Jun. Cellular proteins (10 μg per lane) were separated by 12 % SDS-PAGE, transferred to Immobilon-P membranes and analyzed with antibodies against c-Jun.
B. The amount of c-Jun was estimated by densitometric analysis and expressed as normalized values relative to the level of c-Jun in the control cells. Average values (±SD) from two independent series of experiments (analyzed in duplicates) are shown.
* - $p < 0.05$ compared to the controls, ** - $p < 0.05$ compared to the corresponding curcumin-untreated cells

To study the effects of UV-C light, cultivation medium was removed and fresh medium (5 mL/culture) was added. After 1 h the medium was removed and to prevent the drying of the cells during irradiation, the amount of fresh medium, which corresponds to 1 mm high medium layer, was added (2.8 mL). Cells were irradiated by Phillips UVT 15W/G15T T8 lamp with emission maximum at 254 nm. The intensity of irradiation was determined using BioBlock VLX-3W dosimeter. Control cell cultures were kept at the room temperature during that time. After the exposure to the radiation (10–15 s), all cell cultures were supplemented with removed medium and returned into the incubator for the next 2, 4 and 24 h.

For treatment with MNNG, cultivation medium was removed and fresh medium (5 mL/culture) was added. After 1 h, MNNG (5 μM) was added into medium. In the control cell, MNNG was omitted. Cells were cultivated for the next 4 or 24 h under standard conditions.

To examine effects of curcumin on expression of galectin-3 and c-Jun, cultivation medium was removed and fresh medium containing 50 μM curcumin (5 mL/culture) was added. In the control cell cultures fresh medium without curcumin was added. Cells were cultivated for 1 h before adding MNNG, or exposure to UV-C light, respectively.

Before analysis, cells were washed twice with ice-cold 0.137 mol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L $Na_2HPO_4 \cdot 7\ H_2O$, 1.4 mmol/L KH_2PO_4, pH = 7.4 (PBS), scraped and centrifuged at 600 × g for 10 min. The cell pellet was lyzed in 1 % Triton X-100 in 5 mM Tris/HCl, pH = 8.0, 15 mM NaCl, 2 % Na-azide, 2 mM phenylmethylsulfonyl fluoride (PMSF).

Western-blot analysis

Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12 % polyacrylamide gels according to Laemmli *(23)*. Separated proteins were transferred onto nitrocellulose membrane using semi-dry transfer technique. Membranes were blocked overnight with 3 % bovine serum albumin

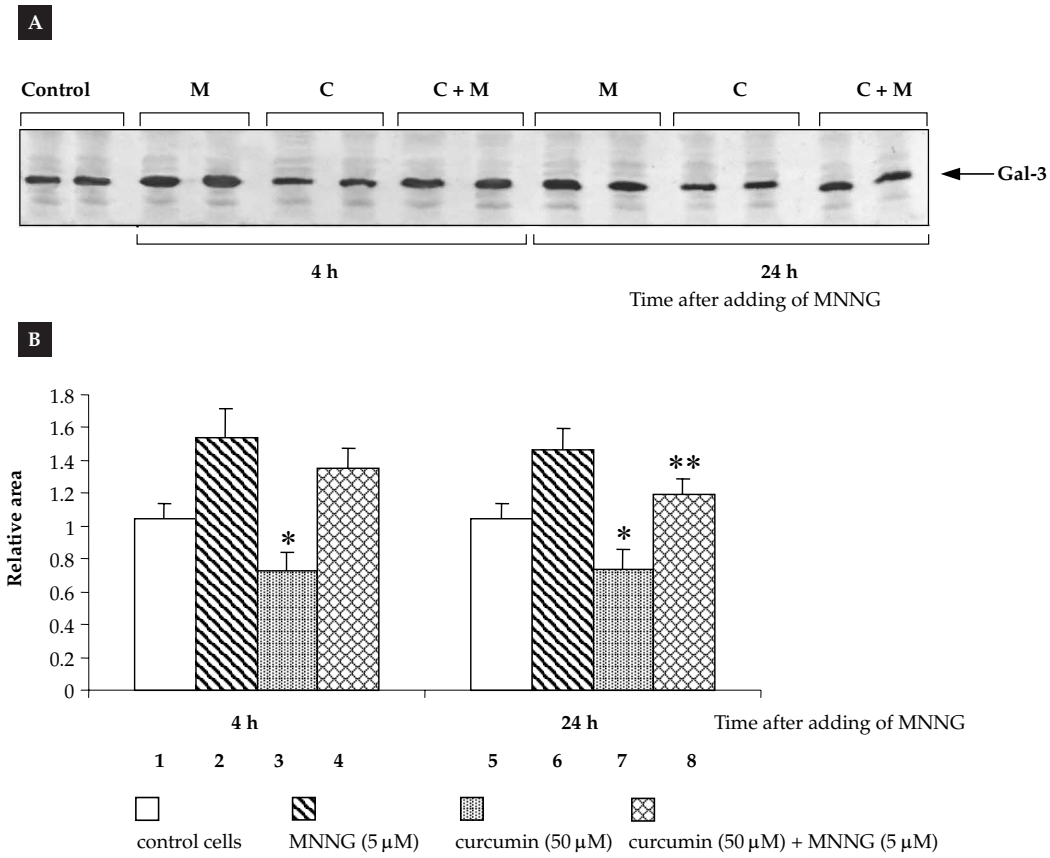

Fig. 3. Effects of curcumin on basal and MNNG-induced expression of galectin-3.
A1235 glioblastoma cells were pre-incubated for 1 h in medium with or without curcumin (50 μM), MNNG was or was not added to the medium (to the final concentration of 5 μM) and analyzed for galectin-3 (Gal-3) after 2, 4 and 24 h of further cultivation. Control cells were exposed neither to curcumin nor to MNNG.
A. Western blot analysis of galectin-3. Cellular proteins (10 μg per lane) were separated by 12 % SDS-PAGE, transferred to Immobilon-P membranes and analyzed with antibodies against galectin-3.
B. The amount of galectin-3 was estimated by densitometric analysis and expressed as normalized values relative to the level of galectin-3 in the control cells. Average values (±SD) from two independent series of experiments (analyzed in duplicates) are shown.
* - $p < 0.05$ compared to the controls; ** - $p < 0.05$ compared to the corresponding curcumin-untreated cells

(BSA) in 0.05 mol/L Tris/HCl, pH = 7.5, 0.15 mol/L NaCl (TBS). Galectin-3 was identified with anti-galectin-3 antibodies (culture supernatant from hibridoma M3/38) diluted 1:50 in 3 % BSA in TBS. c-Jun was identified with polyclonal rabbit anti-Jun antibodies (1:1000 in 3 % BSA in TBS). After incubation with alkaline phosphatase-labeled secondary antibody (goat anti-rat IgG diluted 1:2000 in TBS for galectin-3 and goat anti-rabbit IgG diluted 1:1000 for c-Jun), proteins were visualized with 0.02 g/L 5-bromo-4-chloro-3-indolyl phosphate and 0.04 g/L nitro-blue tetrazolium in 0.05 mol/L Tris/HCl, pH = 9.5, 0.1 mol/L NaCl, 0.005 mol/L $MgCl_2$.

Membranes were incubated in dark until the color developed (15 min on average) and digitalized with UMAX Astra 610P Scanner using 300 × 300 dpi optical resolution. Quantification of the bands corresponding to galectin-3 or c-Jun was performed by two-dimensional analysis (*spot densitometry*) in National Institute of Health (NIH) software (»Tnimage Scientific Image Analysis Software«, by T.J. Nelson). Single factor ANOVA was used to measure statistical significance. Difference of $p < 0.05$ was considered to be statistically significant.

Other procedures

Protein concentrations in cell homogenates were determined by the method of Lowry (*24*).

Results

Effects of curcumin on basal galectin-3 and c-Jun expression

Incubation of A1235 glioblastoma cells with curcumin (50 μM) for 3 h did not result in statistically significant change of galectin-3 level (Fig. 1, bar 5) or c-Jun level (Fig. 2, bar 5). Additional 2 h-incubation resulted in approximately 25 % decrease of galectin-3 level (Fig. 1, bar 6; Fig. 3, bar 3) as well as 20 % decrease of c-Jun level (Fig. 2, bar 6, Fig. 4, bar 3). After prolonged incubation (25 h) galectin-3 level remained in the reached range (Fig. 1, bar 7; Fig. 3, bar 7) while c-Jun level fell further, on approximately 65 % of the initial value (Fig. 2, bar 7; Fig. 4, bar 7).

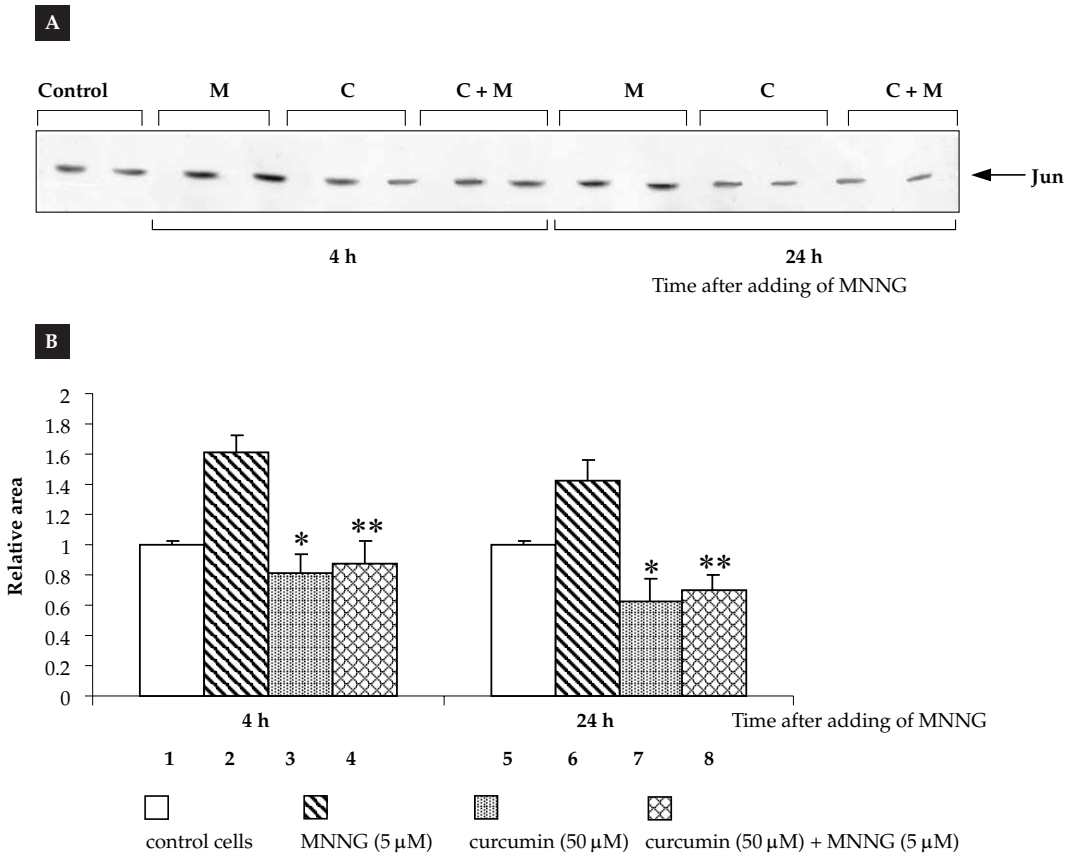

Fig. 4. Effects of curcumin on basal and MNNG-induced expression of c-Jun.
A1235 glioblastoma cells were pre-incubated for 1 h in medium with or without curcumin (50 μM), MNNG was or was not added to the medium (to the final concentration of 5 μM) and analyzed for c-Jun after 2, 4 and 24 h of further cultivation. Control cells were exposed neither to curcumin nor to MNNG.
A. Western blot analysis of c-Jun. Cellular proteins (10 μg per lane) were separated by 12 % SDS-PAGE, transferred to Immobilon-P membranes and analyzed with antibodies against c-Jun.
B. The amount of c-Jun was estimated by densitometric analysis and expressed as normalized values relative to the level of c-Jun in the control cells. Average values (±SD) from two independent series of experiments (analyzed in duplicates) are shown.
* - $p < 0.05$ compared to the controls; ** - $p < 0.05$ compared to the corresponding curcumin-untreated cells

Effects of curcumin on galectin-3 and c-Jun expression in cells exposed to UV-C light

Cell exposure to UV-C radiation of 80 J/m^2 has induced expression of galectin-3. Two hours after irradiation cell content of galectin-3 was approximately 60 % higher compared to the initial values (Fig. 1, bar 2). Further incubation was followed by additional elevation of galectin-3 contents. After 4 and 24 h it reached levels that were 70 % and 80 % higher than the initial one (Fig. 1, bars 3 and 4). At the same time c-Jun levels (2, 4 and 24 h), were found to be increased compared to the controls by 3.7, 6.7 and 4.2 times, respectively (Fig. 2, bars 2, 3 and 4).

Cell pre-exposure to curcumin (50 μM) for 1 h caused considerable suppression of inducible effect of UV-C radiation on galectin-3 expression. Galectin-3 levels in the cells treated with curcumin for 1 h before UV-C exposure were still elevated compared to the controls by 40, 38 and 26 % after 2, 4 and 24 h, respectively (Fig. 1, bars 8, 9 and 10), but after 4 and 24 h they were significantly lower compared to the corresponding curcumin-untreated cells by 20 and 30 %, respectively (Fig. 1, bar 8 *vs.* bar 2, bar 9 *vs.* bar 3, bar 10 *vs.* bar 4). Curcumin pre-treatment had the same effect on c-Jun expression. Although c-Jun levels in curcumin-pretreated UV-C light exposed cells were 2.1, 1.5 and 1.2 times higher compared to the control after 2, 4 and 24 h, respectively (Fig. 2, bars 8, 9 and 10), they were remarkably reduced compared to the cells untreated with curcumin before radiation by 43, 78 and 71 %, respectively (Fig. 2, bar 8 *vs.* bar 2, bar 9 *vs.* bar 3, bar 10 *vs.* bar 4).

Effects of curcumin on galectin-3 and c-Jun expression in cells exposed to MNNG

Alkylating agent MNNG provoked elevation of galectin-3 and c-Jun expression. Within first 4 h after addition of MNNG, galectin-3 amount increased for 54 % compared to the controls (Fig. 3, bar 2) and stayed elevated in the same range for the next 20 h of incubation (Fig. 3, bar 6). Intensity of c-Jun elevation was in the approximately same range. Namely, after 4 h it reached the level that was 63 % higher than the initial one (Fig. 4, bar 2), and after 24 h it was slightly lower, but still 43 % higher than in controls (Fig. 4, bar 6).

When cells were incubated with curcumin for 1 h before exposure to MNNG, inducible effect of MNNG on galectin-3 expression was slightly reduced after 4 h, while after 24 h it was completely abolished. Namely, 4 h after MNNG-exposure galectin-3 level in the curcumin--treated cells was 35 % higher compared to the control (Fig. 3, bar 4), but after 24 h the significant difference compared to the controls was not found (Fig. 3, bar 8). However, the tendency of curcumin to suppress inducible effect of MNNG on galectin-3 expression noticed after 4 h of incubation (Fig. 3, bar 4 *vs.* bar 2) became statistically significant after 24 h of incubation (Fig. 3, bar 8 *vs.* bar 6).

Curcumin had even stronger effect on c-Jun expression. Already after 4 h there was no statistically significant difference between c-Jun level in the cells pretreated with curcumin before MNNG exposure and the control level (Fig. 4, bar 4), while after 24 h c-Jun level was reduced by 30 % compared to the control level (Fig. 4, bar 8). In comparison with the corresponding curcumin untreated cells, cells treaded with curcumin for 1 h before MNNG exposure contained approximately 50 % lower c-Jun amount after 4 and 24 h (Fig. 4, bar 4 compared to bar 2, and bar 8 compared to bar 6).

Discussion

The induction of galectin-3 expression is tightly related to tumorigenicity and malignancy of many kinds of neoplasms *(8–10)* at both the protein and the mRNA levels. Conversely, the inhibition of galectin-3 expression reduces tumor invasiveness and its metastatic potential (*11–13*). Unraveling the interplay of the regulatory factors involved in galectin-3 expression is an important issue *(25–29)* and biomedicine is highly interested in identifying likely inhibitors that restrain the process of galectin-3 expression.

It was shown that in the range of 20 to 200 μM concentrations curcumin, a natural, biologically active compound extracted from turmeric, exhibits a broad spectrum of beneficial biological effects, *e.g.* anti-inflammatory, anti-mutagenic, anti-proliferative, and anti-cancerogenic activities, without any toxic effect *(30–32)*. Although the complete genomic sequence of human galectin-3 gene (*LGALS3*) has been reported, the regulation of *LGALS3* expression is still largely unknown *(33)*. Analysis of the promotor region of *LGALS3* revealed the existence of multiple putative regulatory elements (*e.g.* Sp1, AP-1, NF-κB and cAMP response element binding factor (CREB)). Recently we have shown that the cell exposure to alkylating agent MNNG as well as UV light (λ=254 nm) induce galectin-3 expression and that both transcription factors, Jun (AP-1) and NF-κB take part in the regulatory mechanism of galectin-3 expression *(21)*. These findings, as well as the fact that c-Jun and NF-κB activation can be inhibited by curcumin *(18)*, encouraged us to explore effects of curcumin on galectin-3 expression.

In this work we applied 50 μM concentration of curcumin during 3, 5, and 25 h of incubation. Results showed that it took 5 h of incubation in the presence of curcumin until the decrease of galectin-3 level could be seen. Prolonged incubation of 25 hours did not result in further reduction of galectin-3 level, the 75 % of the control level, achieved after 5 h, was maintained. At the same time c-Jun level, after reduction of 20 % after 5 h continued to fall, and after 25 hours it reached 65 % of the control value. In our earlier studies, Jun was recognized as a regulator of the unstimulated expression of galectin-3, since specific inhibition of *jun* by antisense--*jun* oligonucleotides significantly decreased basal levels of galectin-3, while specific inhibitor of NF-κB, carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone (zL_3-vs) did not show this effect *(21)*. Taken together, these facts suggest that curcumin affects galectin-3 expression in unstimulated cell through transcription factor AP-1, but that some other transcription factor could also be involved in this process, since the decrease of c-Jun level was more pronounced than the decrease of galectin-3.

In this work, we have also shown that curcumin inhibited the elevation of galectin-3 level induced by UV--light radiation and alkylation agent MNNG. Curcumin exerted more significant inhibitory effect when galectin-3 expression was induced with MNNG than with UV-C light. Namely, inducible effect of MNNG was abolished after 24 h, while UV-light-provoked stimulation was not fully eliminated. Similar result was obtained for c-Jun, but it is important to emphasize that UV-C radiation provoked huge elevation of c-Jun level (6.7 times higher level comparing to the controls was detected after 4 hours), while MNNG induced it mostly by 60 % compared to the controls. Such a huge elevation of c-Jun level was not followed by a strong induction of galectin-3. In our previous studies it was noticed that when c-Jun expression was significantly suppressed by antisense-*jun* oligonucleotides, there was still some increase in galectin-3 after the exposure to UV-C light. It was also shown that UV light-induced expression of galectin-3 related transcription factor NF-κB. Curcumin inhibits activation of both of these factors, hence uncompleted suppression of UV light-induced expression of galectin-3 achieved with curcumin suggests that some other factors could be involved in this regulatory mechanism. On the other hand, inducible effect of MNNG on galectin-3 expression was abolished with curcumin treatment, so the central role in regulation of MNNG induced expression of galectin-3 could be attributed to the transcription factors c-Jun and NF-κB.

The key findings of this study include: (*i*) curcumin (50 μM) was found to inhibit basal expression of galectin-3 and c-Jun in human glioblastoma A1235 cells. (*ii*) Cell pretreatment with curcumin (50 μM) for 1 h considerably reduced UV-C light-induced expression of galectin-3 and c-Jun. (*iii*) Cell pretreatment with curcumin (50 μM) for 1 h completely abolished MNNG-induced expression of galectin-3 and c-Jun. This study strongly suggests that curcumin is a potent inhibitor of galectin-3 expression, and that it should be considered as a possible drug for treatment of pathological conditions characterised by elevated expression of galectin-3.

Acknowledgements

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Kurkumin – snažan inhibitor ekspresije galektina-3

Sažetak

Galektin-3, lektin koji specifično prepoznaje β-galaktozidne strukture glikoproteina, sudjeluje u različitim biološkim procesima. Patofiziološka stanja i različiti uzročnici stresa bitno utječu na ekspresiju galektina-3. Premda nisu poznati svi čimbenici u regulacijskom mehanizmu ekspresije galektina-3, pokazano je da u tom procesu sudjeluju transkripcijski faktori AP-1 i NF-κB. Kurkumin, biološki aktivan spoj izoliran iz biljnih podanaka vrste *Curcuma*, interferira s djelovanjem obaju transkripcijskih faktora. Istražili smo učinak kurkumina na ekspresiju galektina-3 u stanicama glioblastoma u bazalnim uvjetima te u stresu koji je bio izazvan izlaganjem stanica alkilirajućem agensu N-metil-N'-nitro-N-nitrozogvanidinu (MNNG) i ultraljubičastom C (UV-C) svjetlu. Razina galektina-3 određena je tehnikom western-blot uz uporabu monoklonskih protutijela M3/38. Utvrđeno je da kurkumin smanjuje bazalnu razinu galektina-3 te da predinkubacija stanica kurkuminom jako smanjuje inducibilni učinak UV-C zračenja, a potpuno uklanja induktivni učinak alkilirajućeg agensa MNNG. Rezultati nedvojbeno pokazuju da je kurkumin snažan inhibitor ekspresije galektina-3.